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(54) Title: METHOD OF PRODUCING OXIDIZED STARCH			
(57) Abstract			
<p>The invention concerns a method for producing oxidized starch. According to the method, the starting material containing starch is contacted with a reagent producing oxoammonium ion, in particular 2,2,6,6-tetramethylpiperidin-1-oxyl, in the presence of an oxidizing agent. According to the invention, an oxidative enzyme, such as laccase, is used as the oxidizing agent. By using laccase as the oxidizing agent is achieved quite selective oxidation while avoiding the halide-containing reagents that are considered harmful to the environment.</p>			

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**Method of producing oxidized starch**

The present invention concerns a method for producing oxidized starch according to the  
5 preamble of claim 1.

According to a method of this kind a starch-containing substrate starch is contacted with a  
reagent that produces oxoammonium ion in the presence of an oxidative agent. The  
reaction is preferably carried out in a liquid medium and the reaction product is separated  
10 from the medium after the reaction, washed and dried.

Starch is a naturally occurring polymer, whose derivatives are used as fillers, for gluing and  
in coating in the paper and board industry. Oxidized starch derivatives as well as starch  
esters and cationized starch are used for surface sizing of paper, for improving strength and  
15 printing properties and as adhesives in coating to bind the pigment particles together and  
onto the base paper.

Industrially, starch is oxidized primarily with the aid of inorganic oxidizing agents, such as  
sodium hypochlorite, the viscosity of the starch dispersion being decreased to a suitable  
20 level and part of the hydroxyl groups of the starch being oxidized to carbonyl and  
carboxylic groups. Uronic acid groups are obtained from the primary hydroxyl groups by  
oxidizing. The carboxylic groups increase the stability of the viscosity of the dispersion.  
As alternative procedures for oxidizing with hypochlorite, the use of hydrogen peroxide,  
bromine, periodate, ozone and oxygen have been proposed, of which oxidation using  
25 hydrogen peroxide is the most promising alternative (1-2).

Conventional oxidation methods are hampered by the problem that the oxidation is not  
specific enough for primary OH-groups. In the chemical oxidation procedures, in addition  
to the primary OH-groups, also the secondary OH-groups are oxidized to some extent. The  
30 oxidation of the secondary OH-groups leads to the breaking of the polymer chain,  
depolymerisation. Because of this more specific methods for oxidizing starch and other  
biosaccharides have been sought for a long time.

The very selective TEMPO derivative represents an interesting alternative for oxidation. The TEMPO radical may, according to literature, be oxidized with the aid of hypohalites, such as hypochlorite and hypobromite. The oxidized TEMPO oxidizes different primary alcohol groups to carboxyls.

5

- The TEMPO-radical (2,2,6,6-tetramethyl piperidine-1-oxyl) has been found to oxidize with a high selectivity the primary OH-groups of carbohydrate polymers. The actual oxidizing agent is the oxoammonium ion (nitrosonium ion), which is obtained by oxidizing TEMPO first with the aid of hypohalites, such as hypochlorite and hypobromite. The  
10 oxoammonium ion oxidizes the primary OH-groups of the carbohydrates to carbonyls which may be oxidized further to carboxyls.

- TEMPO and the derivatives thereof have previously been used for oxidizing the primary hydroxyl groups of alcohols and diols to aldehydes and carboxyls as well as for oxidizing  
15 aliphatic and aromatic aldehydes to carboxyls. In these cases, also additional catalysts have been present and the reaction has taken place in an alkaline two phase system (3-5). TEMPO has also been used for oxidizing different polysaccharides, such as starch in an aqueous phase (6-9). In these cases, the primary oxidizing agent has been the already mentioned hypochlorite and a catalytic amount of sodium bromide, a stronger oxidizing  
20 agent, hypobromite, being produced when these react.

- The method has been stated to exhibit high selectivity and efficiency. Hydrated aldehydes in aqueous solution are produced as intermediates; the aldehydes are further oxidized to carboxyls (7-9).

25

There are also known technical solutions where TEMPO has been oxidized with a superoxide which has been produced with enzymes (12).

- A problem of prior art is that hypochlorite is not only expensive, but also harmful to the  
30 environment. This creates problems when operating on an industrial scale. Enzyme treatment presented in reference (12) is carried out at anaerobic conditions and the technical solution in question is not suitable to large scale production, either.

- It is an object of the present invention to eliminate the problems of the prior art and to provide a completely new method for selective oxidation of starch. In particular, the aim of the present invention is to provide an entirely new process for oxidizing starting materials containing starch by using a reagent which produces an oxoammonium ion, such as
- 5 TEMPO, the regenerating oxidizing agent of the reagent being an oxidizing agent which does not contain halides.

The invention is based on the idea that TEMPO is oxidized with the aid of an oxidizing enzyme. Suitable oxidizing enzymes are phenoloxidases, in particular laccase and

10 peroxidases. Thus, the oxidation of starch can be conducted in a more environmentally friendly way with enzymes and TEMPO than oxidation with chemical oxidizing agents.

More specifically, the technical solution of the present invention is mainly characterized by what is stated in the characterizing part of claim 1.

15 A number of considerable advantages are obtained with the aid of the present invention. Thus, a quite selective oxidation is obtained, both carboxyl and carbonyl groups being obtained in a suitable proportion. The halide-containing materials that are considered environmentally problematic can be eliminated by using phenoloxidase as regenerating

20 oxidizing agent. Oxygen functions as final electron acceptor, when the building up of difficult by-products in the reaction system is avoided, which facilitates the removal and purification of the product. Similarly, when using an oxidizing enzyme, such as phenoloxidase, as regenerating oxidizing agent, mild reaction conditions, such as neutral or almost neutral pH and low temperature may be used.

25 Next, the invention will be more closely examined with the aid of a detailed description and a number of working examples. In the description reference is made to the enclosed drawing, which schematically depicts the reactions of TEMPO in the oxidation system. Figure 1A presents the oxidation of TEMPO resulting from the chemical oxidizing agents

30 and Figure 1B gives a corresponding presentation of a solution according to the present invention, in which the oxidation is obtained by using an enzyme.

As is apparent from Figures 1A and 1B, the stable nitroxyl radical (stage 1) is oxidized to oxoammonium ion (stage 2) which oxidizes the alcohol group ( $\text{RCH}_2\text{OH}$ ) to, e.g.,

aldehyde (RCHO), the oxoammonium ion being at the same time reduced to hydroxyl amine (stage 3).

According to Figure 1A, hypochlorite is used as regenerating oxidizing agent and,  
5 according to Figure 1B, oxygen. In the first case, also a catalytic amount of bromide is brought to the reaction. Bromide oxidizes to hypobromite which is a stronger oxidizing agent than hypochlorite. When using peroxidases, hydrogen peroxide may be used as electron acceptor instead of oxygen.

10 As already mentioned, in the invention, oxidizing (oxidative) enzymes are used. Preferably, enzymes that catalyze the oxidizing of phenolic groups are used. These enzymes are oxidoreductases, such as oxidases and peroxidases. "Oxidases" are enzymes that catalyze oxidation reaction by using molecular oxygen as their substrate while "peroxidases" are enzymes that catalyze oxidation reactions by using hydrogen peroxide as  
15 their substrate. Phenoloxidases (E.C. 1.10.3.2 benzenedioloxygen oxidoreductase) catalyze the oxidation of o- and p- substituted phenolic hydroxyl and amino/amine groups in monomeric and polymeric aromatic compounds.

As specific examples of oxidases the following can be mentioned: laccases (E.C. 1.10.3.2),  
20 cathecoloxidases (E.C. 1.10.3.1), tyrocinases (E.C. 1.14.18.1) and bilirubineoxidases (E.C. 1.3.3.5).

It is particularly preferred to use phenoloxidases, in particular laccases, in the invention.  
Laccases can be produced by white rot fungi, in particular with *Trametes* (previously  
25 *Coriolus*) *versicolor* or *hilluta* or *villosa* strains. Other known micro-organisms producing laccase are the different strains of following mold: *Agaricus*, *Armillaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Lentinus*, *Monocillium*, *Neurospora*, *Phlebia*, *Polyporus*, *Podospora*, *Pycnoporus* and *Schizophyllum*.

30 The invention is, however, not limited to the indicated origins of enzymes nor to a specific isolation method, and the enzymes may be obtained by other means.

Thus it is possible to produce oxidative enzymes with microorganisms that are mutated and genetically built to produce the desired enzyme, or with other host strains to which the genes that code the enzyme are removed.

- 5 The starch to be oxidized may be any natural starch possessing an amylose/amyllopectine ratio of 1:0...0:1. As examples of such sources of starch may be mentioned tuber-bearing plants and cereal such as potato, wheat, barley, oat, pea, corn, rice, sago and tapioca.

The amount of laccase in the oxidation may be approximately 1...1000 nkat/g of starch.

- 10 The concentration of starch may vary on a large range, it may be, e.g., 5...35% (v/w). The oxidation is conducted in the liquid phase, since TEMPO is water soluble preferably in water, at a temperature in the range of 10 to 70 °C, preferably 20 to 40 °C and at a pH in the range of 4 to 9. The amount of TEMPO is 0.01 to 50 wt-%, preferably approximately 0.1 to 20 wt-% of the amount of starch.

15

In practice, the reaction is conducted by dissolving a selected amount of TEMPO (in the form of a stable nitroxyl radical) in water, to which starch is suspended. The dry-content of the suspension is usually set to approximately 0.1 to 20 wt-%. Buffer may be added to water to set the pH of the reaction medium to a desired, suitable in view of laccase

- 20 treatment, value (over 4). After the addition of laccase, oxygen is fed to the reaction mixture, either as gas or as air while simultaneously mixing the composition. Depending on the amount of the material to be oxidized, the feeding of oxygen lasts usually approximately 10 min – 24 h. After the reaction the oxidized starch is separated by filtration from the liquid, washed and dried.

25

As already mentioned, when using peroxidases, TEMPO is oxidized with hydrogen peroxide.

- 30 As a result of oxidation according to the invention starch is obtained, a part of the primary OH-groups of which having been converted to carbonyl and carboxyl groups. Typically, according to the present method the ratio of carbonyl groups to carboxyl groups is 1...5. The molar mass of starch possibly decreases as a result of the oxidation, but is usually still over 300.000 g/mol, e.g., at least 400.000 g/mol.

Oxidized starch can be used as such as an additive in paper making in the wet end of a paper machine or as a coating material. Due to the carbonyl groups, the starch is made reactive and thus the product may also be further oxidized and the carbonyl groups may be further modified.

5

The following non-limiting examples illustrate the invention.

**Example 1 (Comparative example)**

**Chemical oxidation**

10

The oxidation catalyzed by TEMPO was carried out as follows:

0.3 g TEMPO was dissolved in water, 30 g starch was suspended to the solution and 12 g NaBr was added. The temperature of the reaction mixture was adjusted to 0 °C. Another 15 mixture with 345 ml 8 % hypochlorite was prepared, pH was adjusted with 3 M hydrochloric acid to a value of 10.8 and the temperature of the solution was adjusted to 0 °C. The solution was added in one batch to the solution containing starch and incubated for 2 h. During the incubation the pH of the solution was controlled and, if necessary, adjusted back to a value of 10 – 11 with 0.5 M NaOH. The reaction was stopped by adding 60 ml 20 96% ethanol and adjusting thereafter the pH of suspension to 7. The starch was separated and washed with 1000 ml water and dried at room temperature.

**Example 2**

**The lacchase-TEMPO-oxidation of starch**

25

Native potato starch was oxidized by using TEMPO as a intermediate medium and laccase enzyme as an oxidizer.

30

Enzyme: *Trametes hirsuta* D 443 –fungus laccase, growth medium. The activity was defined by using ABTS-substrate (10).

In 0.1 M NaAc buffer a 5 % suspension with a pH of 5.0 was prepared of 35 g native potato starch. TEMPO (3.5 g) was dissolved in the buffer, starch was added and suspended to the liquid phase, after which laccase was added (dosage 200 nkat/g starch). The reaction

was oxidized with an oxygen flow for 3 h at room temperature and simultaneous mixing with a magnetic stirrer. The starch was removed by filtration and washed with 1000 ml water. After washing, the starch was dried at room temperature.

5   **Example 3**

**The properties of oxidized starch**

Carboxyl and carbonyl groups were formed in starch both by chemical oxidation and laccase-TEMPO oxidation. The results are presented in the table below:

10

Starch	COOH/100 glucose unit	CHO/100 glucose unit
native potato starch	0.47 – 0.52	0
chemical TEMPO oxidation	1.07 – 10.3	2.56 – 11.0
laccase-TEMPO oxidation	1.02	2.99

The molecular weights of oxidized starch (both chemically and laccase-TEMPO oxidized) were slightly smaller than the molecular weight of native potato starch.

15   The gelling properties of oxidized starch were determined with Bohl's VOR rheometer (oscillation method). The samples were cooled from 90 °C to 20 °C at a rate of 1 °C/min. The measurements were made in 15 % starch solutions. Laccase-TEMPO and commercial oxidized starch did not gel in the measuring conditions.

20   **Example 4**

Starch was oxidized with different dosages of laccase and TEMPO. The properties of oxidized starch (molecular weight distribution, gelling properties, structure) were determined according to example 3. It was discovered that by varying the dosages of 25   TEMPO and laccase the modification degree of the starch may be altered and thus also the gel properties and molecular weight properties change.

**Literature:**

1. Forssell, P., Hamunen, A., Autio, K., Suortti, T. & Poutanen, K. Starch 47, 371-377 (1995).  
5
2. Parovuori, P., Hamunen, A., Forssell, P., Autio, K. & Poutanen, K. Starch 47, 19-23 (1995).
3. Anelli, P.L., Biffi, C., Montanari, F. & Quici, S. J.Org. Chem. 52, 2559 (1987)  
10
4. Anelli, P.L., Banfi, S., Montanari, F. & Quici, S. J.Org. Chem., 54, 2970 (1989)
5. Kabalka, G.W., Chatla, N., Wadgaonkar, P.P. & Deshpande, S.M. Synth. Comm., 20, 1617 (1990).  
15
6. de Nooy, A.E.J., Besemer, A.C. & van Bekkum, H. Recl. Trav. Chim. Pays-Bas 113, 165-166 (1994).
7. de Nooy, A.E.J., Besemer, A.C. & van Bekkum, H. Carbohydrate Res. 269, 89-98  
20 (1995).
8. Besemer, A.C. & de Nooy, A.E.J. PCT WO 95/07303.
9. Chang, P.S. & Robyt, J.F. J. Carboh. Chem., 15, 819-830 (1996).  
25
10. Niku-Paavola, M.-L., E. Karhunen, P. Salola, and V. Raunio. Biochem. J. 254, 877-884 (1988).
11. Smith, R. J. in Starch: Chemistry and Technology, Eds. R.L. Whistler & E.F. Paschall,  
30 Vol II, Academic Press, Inc., New York 1967, pp. 620-625.
12. Krishna et al., 1992, Proc. Natl. Acad. Sci. USA, Vol 89, pp. 5537-5541

**Claims:**

1. A method of producing oxidized starch, comprising contacting the starting material containing starch with a reagent producing oxoammonium ion in the presence of an oxidizing agent, characterized in that an oxidative enzyme is used as the oxidizing agent.
2. A method according to claim 1, characterized in that phenoloxidase is used as the oxidizing enzyme.
3. A method according to claim 1 or 2, characterized in that the reaction is conducted in liquid medium and the reaction product is separated from the medium after the reaction and conducted to further treatment for purification.
4. A method according to any of claims 1 – 3, characterized in that as the reagent producing oxoammonium ion, 2,2,6,6-tetramethylpiperidin-1-oxyl is used and laccase is used as a regenerating oxidizing agent reacting with it.
5. A method according to any of claims 1 – 4, characterized in that laccase of *Trametes*-, *Agaricus*-, *Armillaria*-, *Aspergillus*-, *Botrytis*-, *Fusarium*-, *Lentinus*-, *Monocillium*-, *Neurospora*-, *Phlebia*-, *Polyporus*-, *Podospora*-, *Pycnoporus*- or *Schizophyllum*- strain is used as peroxidase.
6. A method according to any of claims 1 – 3, characterized in that as the reagent producing oxoammonium ion, 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) is used and peroxidase is used as a regenerating oxidizing agent reacting with it.
7. A method according to any of claims 1 – 3, characterized in that starch is oxidized to obtain aldehyde or carboxyl groups in particular to the primary OH group.
8. Modification of starch with oxidized TEMPO.

9. Modification according to claim 8, characterized in that TEMPO is oxidized enzymatically.
  10. Modification according to claim 8 or 9, characterized in that TEMPO is oxidized with the aid of laccase.
  11. Modification according to claim 8 or 9, characterized in that TEMPO is oxidized with the aid of peroxidase.
- 10 12. The use of modified starch handled with TEMPO as an additive in paper making, in the wet end of a paper machine or as coating material.

## INTERNATIONAL SEARCH REPORT

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PCT/FI 98/00860

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 19/04, C08B 31/18

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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REG, CAPLUS, WPI, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 9507303 A1 (NEDERLANDSE ORGANISATIE VOOR TOEGEPASTNATUURWETENSCHAPPELUK ONDERZOEK TNO), 16 March 1995 (16.03.95), see example 1  --	1-12
Y	WO 9717492 A1 (NOVO NORDISK A/S), 15 May 1997 (15.05.97), claims 1-12  -----	1-12

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Information on patent family members

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WO 9507303 A1	16/03/95	NL	9301549 A	03/04/95
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		CA	2231858 A	15/05/97
		EP	0870088 A	14/10/98